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The Role of the Thomsen-Friedenreich Antigen As a Tumor-Associated Molecule

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The Thomsen-Friedenreich antigen (Gal-GalNAc) represents a tumor-associated molecule, which is assumed to be one of the few chemically well-defined antigens with a proven association with malignancy. In order to analyze the role of the carbohydrate structure Gal-GalNAc for gastrointestinal tumors, we immunized Balb/c mice with MCF-7 breast tumor cells together with synthetic Gal-GalNAc linked to a BSA carrier. One monoclonal antibody (82-A6) was established which recognizes the Thomsen-Friedenreich antigen according to the biochemical and serological analysis presented here. In contrast to the studies performed in the past, immunohistochemical results using this antibody 82-A6 did not exhibit a reactivity clearly restricted to tumors. Preliminary biochemical analysis revealed that the T-determinant is detectable in the high-molecular weight range (about 1000 kD), suggesting that the Gal-GalNAc epitope is found on mucinlike glycoproteins. Tumor restriction of Thomsen-Friedenreich antigen may therefore be determined either by the protein backbone or by the β-glycosidic linkage of the carbohydrate structure to the protein.

Introduction

During carcinogenesis, alterations occur in the biosynthesis of carbohydrate structures on the cell surface. Therefore, carbohydrates linked either to proteins or lipids are recognized as tumor-associated molecules (*1,2*). An interesting novel approach to achieve tumor immunity is to vaccinate patients with chemically synthesized glycopeptides that are restricted to tumors. Several investigators (*3,4*) have succeeded in generating glycopeptides with Gal-GalNAc residues coupled to serine or threonine and bound to a carrier protein. This structure has long been known as the Thomsen-Friedenreich antigen (T-antigen) (*5*). The T-antigen was extensively studied by Springer and his colleagues (*6*) and believed to be tumor restricted. This antigen is the immediate precursor of the human blood group MN antigens, which are located in the NH_2-terminal region of the major sialoglycoprotein (glycophorin A) of human erythrocytes. The immunodominant group of the T-antigen [βGal (1-3) αGalNAc] is described as a cryptic determinant on human erythrocytes, which is exposed by neuraminidase treatment. This unmasked form has been reported to be present on most human carcinomas.

Although it has been assumed that the tumor-associated structure is immunologically similar to the T-antigen expressed on desialylated red blood cells, no one has yet defined the precise carbohydrate group or characterized the molecule carrying the T-antigen determinant on carcinomas. Therefore, we have tried to establish a monoclonal antibody specific for T-antigen.

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Results

An Antibody with Gal-GalNAc Specificity

By immunizing mice with the cultured tumor breast cell line MCF-7 together with a synthetic, chemically synthesized carbohydrate hapten [βGal (1-3) αGalNAc-serine + threonine] linked to a BSA carrier (bovine serum albumin), we succeeded in generating a monoclonal antibody (82-A6, IgM subclass) with T-specificity. Fusion products of spleen cells and cells of the mouse myeloma cell line P3-NS1/1Ag 4-1 were grown in selective media as previously described (*7*). The supernatants were screened for antibody production, and clones were selected on the basis of anti-T reactivity. One clone (mAb 82-A6) was shown to bind to asialoglycophorin, but not to glycophorin, both of which were obtained from Biocarb (Lund, Sweden). Moreover, the antibody reacted with the purified asialoglycoproteins of both the MM and NN blood groups. These asialoglycoproteins differ in the polypeptide chain only.

To make sure that 82-A6 binding to asialoglycophorin is dependent on the carbohydrate residues of the erythrocyte membrane protein, the purified protein was exposed to $NaBH_4$ in alkaline solution to separate the O-glycosidically linked Gal-GalNAc side chains from the

Table 1. Specifity of the (Gal-GalNAc) mAb in comparison to peanut agglutinin.

Antigen source	Reactivity of	
	Anti-T mAb	*Peanut agglutinin*
Asialoglycophorin[a]	Positive	Positive
Glycophorin (bloodgroup MM, NN)	Negative	Negative
Glycophorin, (bloodgroup MM, NN), neuraminidase treated	Positive	Positive
Asialoglycophorin, $NaBH_4$	Negative	Negative
Red blood cells (A,B,0 type)[b]	Weakly positive	Negative
Red blood cells (A,B,0 type), neuraminidase treated	Positive	Positive

[a] Binding of the mAb and peanut agglutinin to enzymatic and chemically treated glycophorin was tested by solid-phase immunosorbent ELISA and by inhibition ELISA after preincubation of mAb with the antigen being tested. MAb- and biotin-conjugated peanut agglutinin binding was visualized by using β-galactosidase-conjugated antimouse immunoglobulin or β-galactosidase conjugated avidin.
[b] For hemagglutination assays, red blood cells were incubated with mAb or peanut agglutinin for 30 min before and after neuraminidase treatment. Agglutination results were scored by visual observation.

protein backbone. MAb binding to asialoglycophorin was abolished after $NaBH_4$ treatment.

Reactivity of the monoclonal antibody was studied by ELISA assays of glycoproteins bound to polyvinyl microtiter plates (*8*). An additional inhibition assay using asialoglycophorin as the target antigen was performed to confirm the results obtained by these direct tests and yielded the same results.

Further analysis included reactivity of mAb 82-A6 with neuraminidase-treated red blood cells. Hemagglutination was obtained with desialylated red blood cells up to a titration end point of 200 ng/mL mAb 82-A6. Agglutination of untreated red blood cells occurred only at very high antibody concentrations and with an incubation period of at least 5 min. Table 1 summarizes the epitope analysis of a mAb 82-A6, demonstrating that the structure recognized represents the T-antigen.

Reactivity of Anti-T mAb with Human Tissues

To determine the tissue reactivity of the mAb 82-A6, normal and malignant tissues were tested as summarized in Table 2. Cryostat sections of frozen specimens were collected on glass slides, air dried, and stained by the indirect immunoperoxidase method. MAb 82-A6 did not show any tumor restriction and stained normal and tumorous epithelial tissues equally well. The antibody also reacted with the majority of cultured human tumor cell lines. In addition to well-known T-positive tumor cell lines, binding sites for anti-T mAb were also found on one of three melanomas. Normal peripheral blood mononuclear cells (PBLs) did not express the T-antigen using mAb 82-A6 by FACS analysis (fluorescent activating cell sorting). T-antigen was not revealed even after treating PBLs with neuraminidase.

Table 2. Reactivity of anti-T mAb with tumor cell lines and tissues.[a]

Targets	Number of positive cell lines/tissues
Cell lines	
Breast, MCF-7, CAMA, BT-20	3/3
Pancreas, Capan-1, -2, ASPC, QGP1	4/10
Stomach, Mz-Sto-1	1/2
Colon, HT-29	1/1
Liver, Mz-Hep-1	1/1
Kidney, SK-RC-6	1/1
Bladder, T-24	1/1
Melanoma, SK-Mel-19	1/3
Epithelial tumor tissues	35/52
Normal epithelial tissues	45/46

[a] Tumor cell lines and cryostat sections obtained from fresh frozen surgical specimen were immunostained with anti-T mAb 82-A6 according to the indirect immunoperoxidase method. As negative control a monoclonal antibody to the mouse T-lymphocyte antigen Lyt 3.2 was used.

Molecular Weight of T-Antigen-Carrying Proteins

Solubilized proteins of the human breast tumor cell line CAMA were applied to a molecular weight exclusion column (Superose 6, Pharmacia, Uppsala, Sweden). Eluted proteins were tested for 82-A6 binding and detected in the high molecular weight fractions of about 1000 kD size. These results suggest that the T-specific carbohydrate epitope is expressed on mucinlike glycoproteins.

Conclusions

In this paper we describe a monoclonal antibody that was generated following immunization of Balb/c mice with a human tumor cell line known to express high amounts of T-antigen together with a chemically synthesized T-structure. The epitope recognized by mAb 82-A6 has been identified as T-antigen on the basis of its reactivity with asialoglycophorin but not glycophorin, its inability to bind deglycosylated asialoglycophorin, and the results of hemagglutination tests with neuraminidase-treated red blood cells.

It appears noteworthy, however, that agglutination of untreated red blood cells also occurred at very high antibody concentrations and after longer incubation periods, which has neither been tested nor observed with any other anti-T antibody. The antibody we describe here reacts with the Gal-GalNac structure, which is linked by an α-glycosidic bond to serine and threonine.

Longenecker et al. have used a panel of α- and β-glycosidically linked Gal-GalNAc structures bound to carrier proteins for immunization (*4*). Their results indicate that only by immunization with the β-glycosidically linked disaccharide, and not with the α-glycosidically linked one, was an mAb with some tumor restriction obtained. These findings are compatible with our own results. Several other investigators have described anti-T mAb (*9–12*). In no case, however, has there been a detailed immunohistochemical analysis to show that the antibodies are tumor restricted. Immunohistochemical studies with mAb 82-A6 revealed that T-antigen is not restricted to malignant tissues but is also found in high amounts on normal tissues. These results suggest that tumor restriction is either determined by the β-glycosidic linkage or depends on the protein backbone as indicated by studies of Schlom and his colleagues of the tumor antigen B72.3 (*13*).

The role of α- or β-glycosidic linkage in relation to tumor association will only be clarified when synthetic carbohydrate structures with definitively known α- or β-linkage to carrier proteins are available. Up to now this has not been the case. Nevertheless, the antibody and the test systems established so far may be useful reagents to detect the real T-antigen, which has a terminal Gal-GalNAc residue.

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